PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/29, 15/82, C07K 14/415, C12N 5/10, A01H 5/00 — A1

(11) International Publication Number: WO 96/38563

(43) International Publication Date: 5 December 1996 (05.12.96)

(21) International Application Number: PCT/US96/08220

(22) International Filing Date: 31 May 1996 (31.05.96)

(30) Priority Data:
08/460,440 2 June 1995 (02.06.95) US

(71) Applicant: PIONEER HI-BRED INTERNATIONAL, INC. [US/US]; 700 Capital Square, 400 Locust Street, Des Moines, IA 50309 (US).

(72) Inventor: RAO, Gururaj, A.; 4734 74th Street, Urbandale, IA 50322-1158 (US).

(74) Agents: SIMON, Soma, G. et al.; 700 Capital Square, 400 Locust Street, Des Moines, IA 50309 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: HIGH METHIONINE DERIVATIVES OF α-HORDOTHIONIN

(57) Abstract

Derivatives of α-hordothionin made by position-specific substitution with methionine residues provide methionine enrichment in plants.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	**GB**	United Kingdom	**MW**	Malawi
AT	Austria	**GE**	Georgia	**MX**	Mexico
AU	Australia	**GN**	Guinea	**NE**	Niger
BB	Barbados	**GR**	Greece	**NL**	Netherlands
BE	Belgium	**HU**	Hungary	**NO**	Norway
BF	Burkina Faso	**IE**	Ireland	**NZ**	New Zealand
BG	Bulgaria	**IT**	Italy	**PL**	Poland
BJ	Benin	**JP**	Japan	**PT**	Portugal
BR	Brazil	**KE**	Kenya	**RO**	Romania
BY	Belarus	**KG**	Kyrgystan	**RU**	Russian Federation
CA	Canada	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SG**	Singapore
CH	Switzerland	**KZ**	Kazakhstan	**SI**	Slovenia
CI	Côte d'Ivoire	**LI**	Liechtenstein	**SK**	Slovakia
CM	Cameroon	**LK**	Sri Lanka	**SN**	Senegal
CN	China	**LR**	Liberia	**SZ**	Swaziland
CS	Czechoslovakia	**LT**	Lithuania	**TD**	Chad
CZ	Czech Republic	**LU**	Luxembourg	**TG**	Togo
DE	Germany	**LV**	Latvia	**TJ**	Tajikistan
DK	Denmark	**MC**	Monaco	**TT**	Trinidad and Tobago
EE	Estonia	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**UG**	Uganda
FI	Finland	**ML**	Mali	**US**	United States of America
FR	France	**MN**	Mongolia	**UZ**	Uzbekistan
GA	Gabon	**MR**	Mauritania	**VN**	Viet Nam

HIGH METHIONINE DERIVATIVES OF α-HORDOTHIONIN

TECHNICAL FIELD

This invention relates to the improvement of feed formulations. Specifically, this invention relates to derivatives of α-hordothionin which provide higher percentages of the essential amino acid methionine in plants.

BACKGROUND OF THE INVENTION

Feed formulations are required to provide animals essential nutrients critical to growth. However, crop plants are generally rendered food sources of poor nutritional quality because they contain low proportions of several amino acids which are essential for, but cannot be synthesized by, animals.

For many years, researchers have attempted to improve the balance of essential amino acids in the proteins of important crops through breeding programs. As more becomes known about storage proteins and the expression of the genes which encode these proteins, and as transformation systems are developed for a greater variety of plants, molecular approaches for improving the nutritional quality of seed proteins can provide alternatives to the more conventional approaches. Thus, specific amino acid levels can be enhanced in a given crop via biotechnology.

One alternative method is to express a heterologous protein of favorable amino acid composition at levels sufficient to obviate food or feed supplementation. For example, a number of seed proteins rich in sulfur amino acids have been identified. A key to good expression of such proteins involves efficient expression cassettes with seed specific promoters. Not only must the gene-controlling regions direct the synthesis of high levels of mRNA, the mRNA must be translated into stable protein.

Among the essential amino acids needed for animal nutrition, often missing from crop plants, are methionine, threonine and lysine. Attempts to increase the levels of these free amino acids by breeding, mutant selection and/or changing the composition of the storage proteins accumulated in crop plants has met with minimal success. Usually, the expression of the transgenic storage protein was too low. The phaseolin-promoted Brazil nut 2S expression cassette is an example of an effective chimeric seed-specific gene. However, even though Brazil nut protein increases the amount of total methionine and bound methionine, thereby improving nutritional value, there appears to be a threshold limitation as to the total amount of methionine that is accumulated in the seeds. The seeds remain insufficient as sources of methionine.

An alternative to the enhancement of specific amino acid levels by altering the levels of proteins containing the desired amino acid is modification of amino acid biosynthesis. Recombinant DNA and gene transfer technologies have been applied to alter enzyme activity catalyzing key steps in the amino acid biosynthetic pathway. Glassman, U.S. Patent No. 5,258,300; Galili, et al.; European Patent Application No. 485970; (1992); incorporated herein in its entirety by reference. However, modification of the amino acid levels in seeds is not always correlated with changes in the level of proteins that incorporate those amino acids. Burrow, et al, Mol. Gen. Genet.; Vol. 241; pp. 431-439; (1993); incorporated herein in its entirety by reference.. Although significant increases in free lysine levels in leaves have been obtained by selection for DHDPS mutants or by expressing the E. coli DHDPS in plants, it remains to be shown that these alterations can increase bound target amino acids, which represent some 90% or more of total amino acids. Thus, there is minimal impact on the nutritional value of seeds.

Based on the foregoing, there exists a need for methods of increasing the levels of the essential amino acids methionine, lysine and threonine in plants.

It is therefore an object of the present invention to provide methods for genetically modifying plants to increase the levels of the essential amino acid methionine in the plants.

It is a further object of the present invention to provide seeds for food and/or feed with higher levels of the essential amino acid methionine than wild species of the same seeds.

DISCLOSURE OF THE INVENTION

It has now been determined that one class of compounds, the ∝-hordothionins, can be modified to enhance their content of methionine. ∝-hordothionin is a 45-amino acid protein which has been well characterized. It can be isolated from seeds of barley (Hordeum vulgare). The molecule is stabilized by four disulfide bonds resulting from eight cysteine residues. The amino acid sequence is as provided in SEQUENCE I.D. No.1. In its native form, the protein is especially rich in arginine and lysine residues, containing 5 residues (10%) of each. However, it is devoid of the essential amino acid methionine.

The protein has been synthesized and the three-dimensional structure determined by computer modeling. The modeling of the protein predicts that the ten charged residues (arginine at positions 5,10,17,19 and 30, and lysine at positions 1,23,32,38 and 45) all occur on the surface of the molecule. The side chains of the polar amino acids (asparagine at position 11, glutamine at position 22 and threonine at position 41) also occur on the surface of the molecule. Furthermore, the hydrophobic amino acids, (such as the side chains of leucine at positions 8,15,24 and 33 and valine at position 18) are also solvent- accessible.

Three-dimensional modeling of the protein indicates that the arginine residue at position 10 is critical to retention of the appropriate 3-dimensional structure and possible folding through hydrogen bond interactions with the C-terminal residue of the protein. A methionine substitution at that point would disrupt the hydrogen bonding involving arginine at position 10, serine at position 2 and lysine at position 45, leading to a destabilization of the structure. The synthetic peptide having this substitution could not be made to fold correctly, which supported this analysis. Conservation of the arginine residue at position 10 provided a protein which folded correctly.

Since methionine is a hydrophobic amino acid, the surface hydrophobic amino acid residues, leucine at positions 8,15, and 33, and valine at position 18, were substituted with methionine. The surface polar amino acids, asparagine at position 11, glutamine at position 22 and threonine at position 41, are substituted with methionine. The resulting compound has the sequence indicated in SEQUENCE I.D. No. 2. The molecule is synthesized by solid phase peptide synthesis and folds into a stable structure. It has seven methoinine residues (15.5%) and, including the eight cysteines, the modified protein has a sulfur amino acid content of 33%.

While SEQUENCE I.D. No. 2 is illustrative of the present invention, it is not intended to be a limitation. Methionine substitutions can also be performed at positions containing charged amino acids. Only arginine at position 10 is critical for maintaining the structure of the protein through a hydrogen-bonding network with serine at position 2 and lysine at position 45. Thus, one can substitute methionine for lysine at positions 1,23,32, and/or 38, and for arginine at positions 5,17,19 and/or 30. The resulting compound has the sequence indicated in SEQUENCE I.D. No. 3.

Synthesis of the compounds is performed according to methods of peptide synthesis which are well known in the

art and thus constitute no part of this invention. In vitro, the compounds have been synthesized on an applied Biosystems model 431a peptide synthesizer using fastmoc™ chemistry involving hbtu [2-(1h-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, as published by Rao, et al., Int. J. Pep. Prot. Res.; Vol. 40; pp. 508-515; (1992); incorporated herein in its entirety by reference. Peptides were cleaved following standard protocols and purified by reverse phase chromatography using standard methods. The amino acid sequence of each peptide was confirmed by automated edman degradation on an applied biosystems 477a protein sequencer/120a pth analyzer. More preferably, however, the compounds of this invention are synthesized in vivo by bacterial or plant cells which have been transformed by insertion of an expression cassette containing a synthetic gene which when transcribed and translated yields the desired compound. Such empty expression cassettes, providing appropriate regulatory sequences for plant or bacterial expression of the desired sequence, are also well-known, and the nucleotide sequence for the synthetic gene, either RNA or DNA, can readily be derived from the amino acid sequence for the protein using standard reference texts. Preferably, such synthetic genes will employ plant-preferred codons to enhance expression of the desired protein.

Industrial Applicability

The following description further exemplifies the compositions of this invention and the methods of making and using them. However, it will be understood that other methods, known by those of ordinary skill in the art to be equivalent, can also be employed.

Plants

The genes which code for these compounds can be inserted into an appropriate expression cassette and introduced into cells of a plant species. Thus, an especially preferred embodiment of this method involves inserting into the genome of the plant a DNA sequence

coding for a compound of this invention in proper reading frame, together with transcription initiator and promoter sequences active in the plant. Transcription and translation of the DNA sequence under control of the regulatory sequences causes expression of the protein sequence at levels which provide an elevated amount of the protein in the tissues of the plant.

Preferred plants that are to be transformed according to the methods of this invention are cereal crops, including maize, rye, barley, wheat, sorghum, oats, millet, rice, triticale, sunflower, alfalfa, rapeseed and soybean.

Synthetic DNA sequences can then be prepared which code for the appropriate sequence of amino acids, and this synthetic DNA sequence can be inserted into an appropriate plant expression cassette.

Likewise, numerous plant expression cassettes and vectors are well known in the art. By the term "expression cassette" is meant a complete set of control sequences including initiation, promoter and termination sequences which function in a plant cell when they flank a structural gene in the proper reading frame. Expression cassettes frequently and preferably contain an assortment of restriction sites suitable for cleavage and insertion of any desired structural gene. It is important that the cloned gene have a start codon in the correct reading frame for the structural sequence.

In addition, the plant expression cassette preferably includes a strong constitutive promoter sequence at one end to cause the gene to be transcribed at a high frequency, and a poly-a recognition sequence at the other end for proper processing and transport of the messenger RNA. An example of such a preferred (empty) expression cassette into which the cDNA of the present invention can be inserted is the pPHI414 plasmid developed by Beach, et al., of Pioneer Hi-Bred International, Inc., Johnston, IA, as disclosed in U.S. patent application No. 07/785,648; (1991); incorporated herein in its entirety by reference.

Highly preferred plant expression cassettes will be designed to include one or more selectable marker genes, such as kanamycin resistance or herbicide tolerance genes.

By the term "vector" herein is meant a DNA sequence which is able to replicate and express a foreign gene in a host cell. Typically, the vector has one or more endonuclease recognition sites which may be cut in a predictable fashion by use of the appropriate enzyme such vectors are preferably constructed to include additional structural gene sequences imparting antibiotic or herbicide resistance, which then serve as markers to identify and separate transformed cells. Preferred markers/selection agents include kanamycin, chlorosulfuron, phosphonothricin, hygromycin and methotrexate. A cell in which the foreign genetic material in a vector is functionally expressed has been "transformed" by the vector and is referred to as a "transformant."

A particularly preferred vector is a plasmid, by which is meant a circular double-stranded DNA molecule which is not a part of the chromosomes of the cell.

As mentioned above, both genomic and cDNA encoding the gene of interest may be used in this invention. The vector of interest may also be constructed partially from a cDNA clone and partially from a genomic clone. When the gene of interest has been isolated, genetic constructs are made which contain the necessary regulatory sequences to provide for efficient expression of the gene in the host cell. According to this invention, the genetic construct will contain (a) a first genetic sequence coding for the protein or trait of interest and (b) one or more regulatory sequences operably linked on either side of the structural gene of interest. Typically, the regulatory sequences will be selected from the group comprising of promoters and terminators. The regulatory sequences may be from autologous or heterologous sources.

Promoters that may be used in the genetic sequence include NOS, OCS and CaMV promoters.

An efficient plant promoter that may be used is an overproducing plant promoter. Overproducing plant promoters that may be used in this invention include the promoter of the cholorophyll α-β binding protein, and the promoter of the small sub-unit (ss) of the ribulose-1,5-biphosphate carboxylase from soybean. See e.g. Berry-Lowe, et al., J. Molecular and App. Gen.; Vol. 1; pp. 483-498; (1982); incorporated herein in its entirety by reference. These two promoters are known to be light-induced, in eukaryotic plant cells. See e.g., An Agricultural Perspective, A. Cashmore, Pelham, New York, 1983, pp. 29-38, G. Coruzzi, et al., J. Biol. Chem., Vol. 258; p. 1399 (1983), and P. Dunsmuir, et al., J. Molecular and App. Gen., Vol. 2; p. 285 (1983); all incorporated herein in their entirety by reference.

The expression cassette comprising the structural gene for the protein of this invention operably linked to the desired control sequences can be ligated into a suitable cloning vector. In general, plasmid or viral (bacteriophage) vectors containing replication and control sequences derived from species compatible with the host cell are used. The cloning vector will typically carry a replication origin, as well as specific genes that are capable of providing phenotypic selection markers in transformed host cells. Typically, genes conferring resistance to antibiotics or selected herbicides are used. After the genetic material is introduced into the target cells, successfully transformed cells and/or colonies of cells can be isolated by selection on the basis of these markers.

Typically, an intermediate host cell will be used in the practice of this invention to increase the copy number of the cloning vector. With an increased copy number, the vector containing the gene of interest can be isolated in significant quantities for introduction into the desired plant cells. Host cells that can be used in the practice of this invention include prokarvotes, including bacterial

hosts such as E. coli, S. typhimurium, and Serratia marcescens. Eukaryotic hosts such as yeast or filamentous fungi may also be used in this invention. Since these hosts are also microorganisms, it will be essential to ensure that plant promoters which do not cause expression of the protein in bacteria are used in the vector.

The isolated cloning vector will then be introduced into the plant cell using any convenient technique, including electroporation (in protoplasts), retroviruses, bombardment, and microinjection into cells from monocotyledonous or dicotyledonous plants in cell or tissue culture to provide transformed plant cells containing as foreign DNA at least one copy of the DNA sequence of the plant expression cassette. Preferably, the monocotyledonous species will be selected from maize, sorghum, wheat or rice, and the dicotyledonous species will be selected from soybean, alfalfa, rapeseed, sunflower or tomato. Using known techniques, protoplasts can be regenerated and cell or tissue culture can be regenerated to form whole fertile plants which carry and express the gene for a protein according to this invention. Accordingly, a highly preferred embodiment of the present invention is a transformed maize plant, the cells of which contain as foreign DNA at least one copy of the DNA sequence of an expression cassette of this invention.

It will also be appreciated by those of ordinary skill that the plant vectors provided herein can be incorporated into agrobacterium tumefaciens, which can then be used to transfer the vector into susceptible plant cells, primarily from dicotyledonous species. Thus, this invention provides a method for increasing methionine levels in agrobacterium tumefaciens-susceptible dicotyledonous plants in which the expression cassette is introduced into the cells by infecting the cells with agrobacterium tumefaciens, a plasmid of which has been modified to include a plant expression cassette of this invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Pioneer Hi-Bred International, Inc.

(ii) TITLE OF INVENTION: High Methionine Derivatives of Alpha-Hordothionin

(iii) NUMBER OF SEQUENCES: 3

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Pioneer Hi-Bred International, Inc.
(B) STREET: 700 Capital Square, 400 Locust Street
(C) CITY: Des Moines
(D) STATE: Iowa
(E) COUNTRY: United States of America
(F) ZIP: 50309

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: PCT
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Simon, Soma G.
(B) REGISTRATION NUMBER: 37,444
(C) REFERENCE/DOCKET NUMBER: 355-US

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 515-248-4896
(B) TELEFAX: 515-248-4844

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Lys Ser Cys Cys Arg Ser Thr Leu Gly Arg Asn Cys Tyr Asn Leu Cys

1 5 10 15

Arg Val Arg Gly Ala Gln Lys Leu Cys Ala Gly Val Cys Arg Cys Lys
20 25 30

Leu Thr Ser Ser Gly Lys Cys Pro Thr Gly Phe Pro Lys
35 40 45

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Lys Ser Cys Cys Arg Ser Thr Met Gly Arg Met Cys Tyr Asn Met Cys
1 5 10 15

Arg Met Arg Gly Ala Met Lys Leu Cys Ala Gly Val Cys Arg Cys Lys
20 25 30

Met Thr Ser Ser Gly Lys Cys Pro Met Gly Phe Pro Lys
35 40 45

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Ser Cys Cys Met Ser Thr Met Gly Arg Met Cys Tyr Asn Met Cys
1 5 10 15

Met Met Met Gly Ala Met Met Met Cys Ala Gly Val Cys Met Cys Met
20 25 30

Met Thr Ser Ser Gly Met Cys Pro Met Gly Phe Pro Lys
35 40 45

WHAT IS CLAIMED IS:

1.

A protein having the sequence of SEQUENCE I.D. No. 3 wherein the amino acid residues at one or more of positions 1,5,8,11,15,17,18,19,22,23,24,30,32,33,38, and 41 are methionine, and the remainder of the residues at those positions are the residues at the corresponding positions in SEQUENCE I.D. No. 1.

2.

The protein of Claim 1 wherein one or more of the amino acid residues at positions 8,11,15,18,22,33 and 41 are methionine.

3.

The protein of Claim 2 wherein at least 3 of the amino acid residues at positions 8,11,15,18,22,33 and 41 are methionine.

4.

The protein of Claim 3 wherein at least 5 of the amino acid residues at positions 8,11,15,18,22,33 and 41 are methionine.

5.

A nucleotide sequence which codes for a protein having the sequence of SEQUENCE I.D. No. 2 wherein the amino acid residues at one or more of positions 1,5,8,11,15,17,18,19,22,23,24,30, 32,33,38,and 41 are methionine, and the remainder of the residues at those positions are the residues at the corresponding positions in SEQUENCE I.D. No. 1.

6.

An RNA sequence which codes for a protein having the sequence of SEQUENCE I.D. No. 2 wherein the amino acid residues at one or more of positions 1,5,8,11,15,17,18,19,22,23,24,30, 32,33,38,and 41 are methionine, and the remainder of the residues at those

positions are the residues at the corresponding positions in SEQUENCE I.D. No. 1.

7.

A DNA sequence which codes for a protein having the sequence of SEQUENCE I.D. No. 3 wherein the amino acid residues at one or more of positions 1,5,8,11,15,17,18,19,22,23,24,30,32,33,38, and 41 are methionine, and the remainder of the residues at those positions are the residues at the corresponding positions in SEQUENCE I.D. No. 1.

8.

An expression cassette containing the DNA sequence of Claim 7 operably linked to plant regulatory sequences which cause the expression of the DNA sequence in plant cells.

9.

A bacterial transformation vector comprising an expression cassette according to Claim 8, operably linked to bacterial expression regulatory sequences which cause replication of the expression cassette in bacterial cells.

10.

Bacterial cells containing as a foreign plasmid at least one copy of a bacterial transformation vector according to Claim 9.

11.

Transformed plant cells containing at least one copy of the expression cassette according to Claim 8.

12.

The transformed plant cells of Claim 11, wherein the cells are of a monocotyledonous species.

13.

The transformed plant cells of Claim 12, wherein the cells are selected from the group consisting of maize, sorghum, wheat and rice cells.

14.

The transformed cells of Claim 11, wherein the cells are of a dicotyledonous species.

15.

The transformed cells of Claim 14, wherein cells are selected from the group consisting of soybean, alfalfa, rapeseed, sunflower, tobacco and tomato cells.

16.

A maize cell or tissue culture comprising cells according to Claim 13.

17.

A method for enhancing the methionine content of a plant cell or seed comprising the step of expressing a protein according to Claim 1 in the cell or seed.

18.

The method of Claim 17 wherein the plant is a dicotyledonous plant.

19.

The method of Claim 17 wherein the plant is a monocotyledonous plant.

20.

The method of Claim 19 wherein the methionine content of the plant seed is enhanced.

INTERNATIONAL SEARCH REPORT

Interr nal Application No
PC1/US 96/08220

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/29 C12N15/82 C07K14/415 C12N5/10 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	THE PLANT JOURNAL, vol. 3, no. 5, 1993, pages 721-727, XP002015695 KARCHI, H., ET AL.: "Seed-specific expression of a bacterial desensitized aspartate kinase increases the productionof seed threonine and methionine in transgenic tobacco" see page 726, left-hand column, line 33 - line 37 ---	1,2,5-19
Y	WO,A,94 16078 (PIONEER HI BRED INT) 21 July 1994 see the whole document --- -/--	1,2,5-19

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 October 1996	28.10.96
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer Maddox, A

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PROTEIN ENGINEERING, vol. 7, no. 12, 1 December 1994, pages 1485-1493, XP000482988 RAO A G ET AL: "STRUCTURE-FUNCTION VALIDATION OF HIGH LYSINE ANALOGS OF -HORDOTHIONIN DESIGNED BY PROTEIN MODELING" see the whole document	1-20
A	PLANT MOLECULAR BIOLOGY, vol. 24, 1994, pages 83-96, XP002015812 FLORACK, D.E.A., ET AL.: "Expression of biologically active hordothionins in tobacco. Effects of pre- and pro-sequences at the amino and carboxyl termini of the hordothionin precursor on mature protein expression and sorting" see the whole document	8,11-16
A	WO,A,93 03160 (DU PONT) 18 February 1993 see the whole document	1-20
A	WO,A,89 04371 (UNIV LOUISIANA STATE) 18 May 1989 see page 18, line 25 - page 24, line 10	1-20
A	EP,A,0 318 341 (PLANT GENETIC SYSTEMS NV) 31 May 1989 see the whole document	1-20
A	WO,A,94 10315 (PIONEER HI BRED INT) 11 May 1994 see page 14, line 1 - line 14	1-20
A	TRENDS IN BIOTECHNOLOGY, vol. 8, no. 6, 1990, pages 156-160, XP002015696 ALTENBACH, S.B., ET AL.: "Manipulation of methionine-rich protein genes in plant seeds" see the whole document	1-20
A	PLANT MOLECULAR BIOLOGY, vol. 18, 1992, pages 235-245, XP002015698 ALTENBACH, S.B., ET AL.: "Accumulation of a Brazil nut albumin in seeds of transgenic canola results in enhanced levels of seed protein methionine" see the whole document	1-20
A	WO,A,94 20628 (DU PONT) 15 September 1994 see the whole document	15,17,20

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,92 14822 (DU PONT) 3 September 1992 see example 12 ---	16,19
A	J. CELL. BIOCHEM. SUPPL., vol. 16f, 1992, page 225 XP002015780 ANDERSON, J., ET AL.: "A transgenic corn line with altered levels of a high-methionine storage protein" see abstract Y304 -----	16,19

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 96/08220

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9416078	21-07-94	AU-A-	6162294	15-08-94
		CA-A-	2161881	21-07-94
WO-A-9303160	18-02-93	AU-B-	661334	20-07-95
		AU-A-	2441292	02-03-93
		CA-A-	2114788	18-02-93
		EP-A-	0598806	01-06-94
		JP-T-	7502163	09-03-95
		ZA-A-	9205984	10-02-94
WO-A-8904371	18-05-89	AU-A-	2802989	01-06-89
		CA-A-	1321157	10-08-93
		EP-A-	0675960	11-10-95
EP-A-0318341	31-05-89	CA-A-	1337048	19-09-95
		AU-B-	634987	11-03-93
		AU-A-	4495189	01-05-90
		CA-A-	2000661	14-04-90
		WO-A-	9004032	19-04-90
		JP-T-	3502644	20-06-91
		AU-A-	2811889	23-05-89
		WO-A-	8903887	05-05-89
		EP-A-	0319353	07-06-89
		JP-T-	2501802	21-06-90
		US-A-	5487991	30-01-96
		AT-T-	140959	15-08-96
		DE-D-	3855455	05-09-96
		EP-A-	0723019	24-07-96
WO-A-9410315	11-05-94	AU-A-	5446694	24-05-94
WO-A-9420628	15-09-94	AU-A-	6355594	26-09-94
		CA-A-	2158018	15-09-94
		EP-A-	0687303	20-12-95
		JP-T-	8507219	06-08-96
		PL-A-	310524	27-12-95
WO-A-9214822	03-09-92	AU-B-	670409	18-07-96
		AU-A-	1353892	15-09-92
		BG-A-	98042	30-06-94

INTERNATIONAL SEARCH REPORT

...formation on patent family members

Inter...nal Application No
PC...1/US 96/08220

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9214822		CA-A- 2104022	15-08-92
		CZ-A- 9301679	16-03-94
		EP-A- 0571500	01-12-93
		HU-A- 68405	28-06-95
		JP-T- 6505387	23-06-94
		SK-A- 87893	12-01-94

Form PCT/ISA/210 (patent family annex) (July 1992)